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Morphological and mitochondrial-DNA variation in *Rhinolophus rouxii* (Chiroptera)

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Abstract. A systematic review of the rufous horseshoe bat *Rhinolophus rouxii* was undertaken using morphological data recorded from external, cranial and dental characters and sequence data of the cytochrome b gene of mitochondrial DNA. Individuals of the currently recognised subspecies *R. rouxii rouxii*, *R. rouxii sinicus* and *R. rouxii rubidus* were examined from throughout the range. Data from twenty-two morphological characters were used in multivariate statistical analyses. Molecular data were analysed using parsimony methods. All of the analyses showed a high level of concordance in establishing that *R. sinicus* represents a discrete species. Individuals from Sri Lanka are provisionally referred to *R. r. rubidus*.

Key words. *Rhinolophus rouxii*, *Rhinolophus sinicus*, morphology, mitochondrial DNA, Asia.

Introduction

The family Rhinolophidae Bell 1836 (sensu Corbet & Hill 1992) consists of a single genus, *Rhinolophus* Lacépède, 1799 and is currently considered to include 64 species (Koopman 1993). It has an extensive geographical range. Rhinolophid bats are found throughout the Old World from Europe to Japan, through Africa, south-east Asia, the Philippines, New Guinea and Australia (Corbet & Hill 1992). The first comprehensive review of *Rhinolophus* was included in the two papers by Andersen (1905b, 1918) in which the genus was divided into six groups: *megaphyllus*, *pusillus*, *hipposideros*, *luctus*, *macrotis* and *euryotis*. Tate & Archbold (1939) listed Andersen's synoptic arrangement and updated his groupings to include species and subspecies described since 1918. The *megaphyllus* group was renamed the *ferrumequinum* group. Tate (1943) further modified these groups, dividing the *philippinensis* group into three sections, *philippinensis*, *trifoliatus* and *luctus*, an arrangement which was followed by Ellerman & Morrison-Scott (1951). Recent research includes phylogenetic analysis of the family Rhinolophidae by Bogdanowicz & Owen (1992), and a taxonomic listing by Koopman (1993).

R. rouxii, a member of the *ferrumequinum* group (Tate & Archbold 1939), was described by Temminck (1835) with the type locality listed as Calcutta and Pondicherry, India but restricted to Calcutta by Andersen (1905a). Andersen (1905a) named *R. rouxii sinicus* from Chinteh, China and included *R. rubidus*, *R. cineracens* and *R. rammanika* from Sri Lanka and *R. petersii* from India as synonyms of *R. rouxii*. This view was subsequently followed by Tate and Archbold (1939), Ellerman and Morrison-Scott (1951) and Sinha (1973) and more recently by Corbet & Hill (1992) and Koopman (1993). Bates & Harrison (1997) extended the range of *R. r.*

sinicus to include the Himalayas and Nepal, and additionally recognised the subspecies *R. r. rubidus* Kelaart, 1850 from Sri Lanka.

Recently, a study of the bat fauna of the Indian Subcontinent was undertaken by the Harrison Zoological Museum in collaboration with the Bombay Natural History Society and the Department of National Museums Colombo. During this study, variation was recorded between Himalayan individuals of *R. rouxii*, currently referred to *R. r. sinicus* (sensu Bates & Harrison 1997) and those from peninsular India and Sri Lanka, currently referred to *R. r. rouxii*. The degree of variation observed was greater than that usually found between subspecies, and as such it was hypothesised that *R. rouxii sinicus* may represent a distinct species. The proposed hypothesis was subsequently tested, and a comprehensive taxonomic review of *R. rouxii* from throughout its geographic range carried out using morphometric and molecular analyses.

Materials and Methods

Morphological

In total, 172 adult specimens assigned to *R. rouxii* were examined for this study, listed in "Species diagnoses". The material, in the form of study skins and skulls, or fluid preserved specimens, was held in the collections of the Harrison Zoological Museum, Sevenoaks (HZM) or was loaned from natural history museums in Europe, North America and the Indian Subcontinent. These included the Natural History Museum, London (BM); Museum National D'Histoire Naturelle, Paris (CG); The Hungarian Museum of Natural History, Budapest (HM); American Museum of Natural History, New York (AMNH); and the Bombay Natural History Society (IN / MM). In addition, a number of voucher specimens were collected personally on 3 field trips to Tamil Nadu, Karnataka and Uttar Pradesh in India and Southern Province in Sri Lanka. The majority were caught in a hand-held butterfly net whilst the bats were resting in their diurnal roosts, such as caves, mosques and farm buildings, whilst others were collected in Japanese mist-nets erected in open sites. They were prepared as either dry skins and skulls, or as wet specimens preserved in 70% ethyl alcohol.

Morphological characters were examined in all specimens in order to provide detailed diagnostic descriptions of taxa. In addition, bacula were prepared where possible, as described by Thomas et al. (1994). Measurements were taken of twenty-six morphological characters, as described by Bates & Harrison (1997), using dial calipers accurate to 0.1 mm. Of these, twenty-two characters (11 post-cranial, 8 cranial and 3 dental) were used in the multivariate statistical analyses. They are listed below and correspond to those of Bogdanowicz and Owen (1992) who studied the phylogeny of the genus *Rhinolophus*.

1) Forearm length (FA)
2) Length of 5th metacarpal (5MET)
3) Length of 4th metacarpal (4MET)
4) Length of 3rd metacarpal (3MET)
5) Length of 2nd metacarpal (2MET)
6) Length of 1st phalanx of 5th finger (1V)
7) Length of 2nd phalanx of 5th finger (2V)
8) Length of 1st phalanx of 4th finger (1IV)
9) Length of 2nd phalanx of 4th finger (2IV)
10) Length of 1st phalanx of 3rd finger (1III)
11) Length of 2nd phalanx of 3rd finger (2III)
12) Condylocanine length (CCL)
13) Palatal length (PL)
14) Maxillary breadth (M^3-M^3)
15) Upper tooth row (C^1-M^3)
16) Anterior palatal width (C^1-C^1)
17) Post orbital breadth (POB)
18) Zygomatic breadth (ZB)
19) Mastoid breadth (MB)
20) Basioccipital width (BOW)
21) Mandible length (ML)
22) Mandibular tooth row (C_1-M_3)

Complete data for each of the twenty-two characters listed were recorded for 113 of the 172 individuals measured. This data set was divided into operational taxonomic units (OTU's) for

use in the multivariate analyses. It was necessary to pool specimens from several localities into one OTU, which were defined on the basis of collecting gaps and potential physiographic barriers. In order to avoid the formation of OTU's with geographic variation within them, each proposed OTU was checked for within-group geographic variation by single-linkage cluster analysis of cases. If a proposed OTU appeared to be heterogeneous it was split accordingly.

Geographical variation was examined by undertaking discriminant analysis. This procedure generated a set of discriminant functions which provide the best discrimination between the OTU's analysed. Euclidean distances were calculated between each OTU from standardised variables. All analyses were undertaken using SPSS Base 8.0. All twenty-two characters were included in the analyses. Males and females were analysed separately to eliminate any variation due to sexual dimorphism. Specimens included in the discriminant analyses are listed in "Species diagnoses".

Molecular

Samples of wing membrane for molecular analysis were collected from nine specimens of *R. rouxii* and one specimen of *R. ferrumequinum* currently held in the collections of the Harrison Zoological Museum, Sevenoaks (HZM). Wing punches were collected from each specimen using 8mm sterile biopsy punches and stored in tissue collection buffer (6M NaCl, 20% DMSO [Dimethylsulphoxide]) at −20°C. Due to a lack of available material, DNA of *R. rouxii* from Myanmar and China was not sequenced. Specimens used in the analyses are listed in "Species diagnoses".

The DNA was extracted from the wing membrane using a standard protinase K digestion method involving chloroform extractions (Worthington-Wilmer & Barratt 1996). Using the polymerase chain reaction (PCR) (Mullis & Faloona 1987), two segments of mtDNA were amplified with the universal primers H15149 (Kocher et al. 1989), L14724, H15915 and L15513 (Irwin et al. 1991). The primer names refer to the 3' position of the primers relative to human mtDNA light (L) or heavy (H) strands (Anderson et al. 1981). PCR amplifications were performed in 50 ml reaction volumes containing 2 ml deoxynucleoside triphosphate mix (dATP, dCTP, dGTP and dTTP), 5 ml of Bioline KCl buffer, 0.3 ml of each primer (concentration 25 pM), 0.1 ml of Taq polymerase and 2 ml template DNA, made up to a final volume of 50 ml with sterile double distilled water. The amplification cycle was 94°C for 30 s, 45°C for 45 s and 72°C for 45 s. Reactions were run for 32 cycles.

Sequencing was carried out using the Sanger (Sanger et al. 1977) direct double-stranded sequencing method described by Hillis et al. (1996). Two modifications to the method were made, namely the addition of DMSO to the sequencing reaction to overcome problems related to template reannealing (Winship 1989), and the use of Sequenase (Version 2.0 kit US Biomedical) as the DNA polymerase (Green et al. 1989). DNA sequences were read into Macvector version 4.0, and multiple sequence alignment was carried out using DAPSA version 3.8 (Harley 1995). A single data set of 525 bp per individual was constructed of aligned sequence data.

The character-based approach of parsimony analysis was used to construct phylogenetic trees using PAUP (phylogenetic analysis using parsimony) version 3.0 for the Apple Macintosh (Swofford 1990) and HENNIG86 version 1.5 for IBM (Farris 1988). Exact searches were undertaken in PAUP using the branch-and-bound algorithm. The reliability of branches on the trees obtained was estimated by performing a bootstrap analysis using a branch-and-bound search with 100 replications. In HENNIG86, variable nucleotide positions were treated as unordered discrete characters. Trees were calculated using implicit enumeration, and in addition, the successive-approximations approach to character weighting was used, with the process being continued until the same tree was obtained on two successive passes (Farris 1969). Confidence in tree topology was further assessed using Jac (Farris et al. 1996), a program which performs random resampling on molecular data sets to produce a tree showing confidence frequencies in nodes. Jac replications were set at 10000. *Rhinolophus ferrumequinum* from the UK was used as the outgroup in all analyses.

Results

Morphological

Ten OTU's were designated for the 113 individuals analysed (Table 1). Discriminant analysis undertaken on males indicates clearly that the populations cluster into four distinct groups, between which there is no overlap (Fig. 1). Cluster A comprises individuals of *R. rouxii* from Sri Lanka, the states of Goa, Karnataka, Maharashtra and Orissa in peninsular India and southern Myanmar, and is characterised by positive values along the first discriminant function. Cluster B comprises *R. rouxii* from Mysore in Karnataka and from the state of Tamil Nadu, southern India and is characterised by positive values along the second discriminant function. Cluster C comprises of *R. rouxii* from the state of Uttar Pradesh, northern India and Nepal. Cluster D comprises of *R. rouxii* from China. Both clusters C and D are characterised by negative values along both discriminant functions. The first two discriminant functions account for 84.7% of the total variance. A geographical representation of each cluster is shown in Fig. 2.

Table 1: Operational taxonomic units (OTU's) and sample sizes (n) used in morphological analyses.

OTU	Locality	n
1	Kalutara, Matale, Pussahena, Ruwanwella – Sri Lanka	18
2	Coonoor, High Wavy Mountains, Mysore, Shevaroy Hills – India	17
3	Kodura – India	2
4	Barchi, Colva, Devikop, Jog Falls, Savantvadi, Sirsi, Supa, Talewadi – India	31
5	Asgani, Bombay, Karnala – India	11
6	Udyagiri – India	10
7	Mussoorie – India	6
8	Godavari, Parchung – Nepal; Darjeeling – India	8
9	Toungoo – Myanmar	1
10	Chungan Hsien, Wanhsien, Yenping – China; Lam Tao Island – Hong Kong	9

Discriminant analysis undertaken on females shows the populations clustering into three distinct groups which do not overlap (Fig. 3). Cluster E comprises of individuals of *R. rouxii* from Sri Lanka, the states of Goa, Karnataka, Maharashtra, Orissa, Andhra Pradesh and Tamil Nadu in peninsular India. This cluster is characterised by positive values along the first discriminant function. Cluster F comprises of individuals of *R. rouxii* from Uttar Pradesh, northern India and Nepal, and is characterised by negative values along the first discriminant function. Cluster G comprises of individuals of *R. rouxii* from China, and is characterised by negative values along the first discriminant function and positive values along the second discriminant function. The first two discriminant functions account for 79.7% of the total variance. A geographical representation of each cluster is given in Fig. 4.

Euclidean distances (Table 2a, b) show populations of *rouxii rouxii* and *rouxii sinicus* to be relatively well separated, with the largest distances in the Table being between the Chinese population (OTU 10) and populations from Myanmar (9), peninsular India (2–6) and Sri Lanka (1). Relatively small distances between the Chinese population and those from northern India (7) and Nepal (8) indicate that

these populations represent a taxon discrete from the other populations examined. Distances between both the Sri Lankan and Koduran (3) populations when compared to all other populations of *R. r. rouxii* examined are relatively great, suggesting possible subspecific variation within this taxon.

Table 2: Euclidean distances between nine OTU's designated for a) male individuals b) female individuals.

a. Males

Operational Taxonomic Unit	OTU 1	OTU 2	OTU 4	OTU 5	OTU 6	OTU 7	OTU 8	OTU 9
1								
2	7.461							
4	4.705	4.107						
5	4.464	4.159	2.817					
6	6.634	3.381	2.826	3.726				
7	6.758	4.313	5.297	5.359	5.566			
8	7.140	6.113	6.803	6.613	6.977	3.235		
9	5.209	6.066	3.523	4.072	5.109	6.662	8.176	
10	9.293	10.433	10.497	10.481	10.621	7.609	5.422	11.476

b. Females

Operational Taxonomic Unit	OTU 1	OTU 2	OTU 3	OTU 4	OTU 5	OTU 6	OTU 7	OTU 8
1								
2	7.225							
3	5.098	8.635						
4	4.448	3.795	5.755					
5	5.100	2.997	7.070	2.610				
6	7.056	3.231	8.792	3.684	2.851			
7	5.545	7.256	6.904	5.205	6.052	6.387		
8	6.276	8.700	7.742	6.291	6.831	6.866	3.390	
10	7.698	11.091	8.708	8.351	9.492	9.797	5.059	4.233

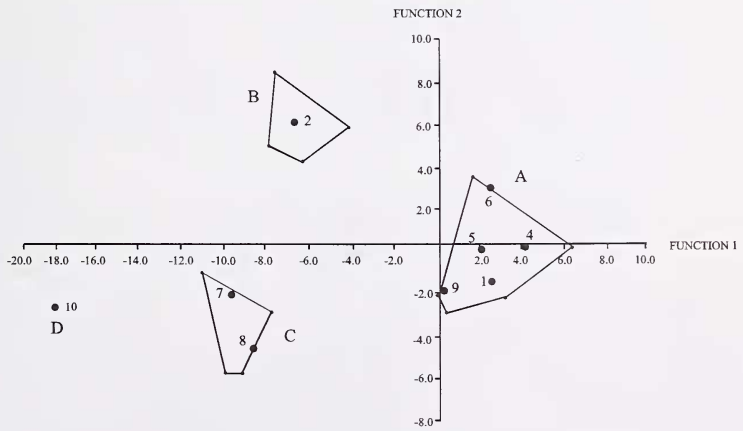

Fig. 1: Ordination of all designated OTU's along the first two discriminant functions from analysis of 56 male specimens. Numbers correspond to OTU means. Lines indicate the extent of scatter of individual specimens. OTU's are listed in Table 1.

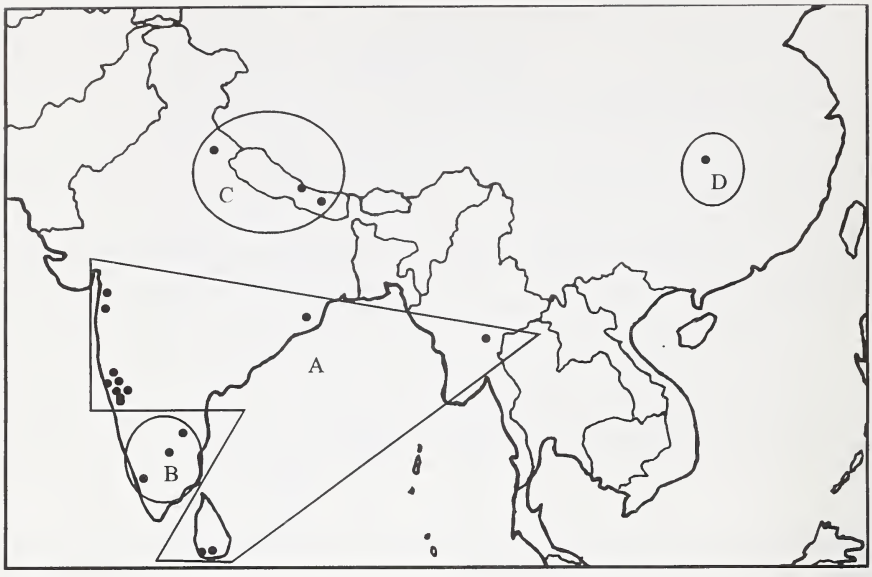

Fig. 2: Geographical representation of clusters A-D from discriminant analysis of male individuals.

Molecular

Results from the mtDNA sequence analyses undertaken showed a similar pattern of variation. Sequence divergence between *R. rouxii* from Sri Lanka and peninsular India and *R. rouxii sinicus* from northern India ranges from 8.6–13.3% (Table 3) (GenBank accession numbers AF109649–AF109652).

Exact searches generated two most parsimonious trees of length 151, consistency index 0.860 and retention index 0.865. There were 64 parsimony informative characters in the data set. The topology of the strict concensus tree (Fig. 5a) was supported by results from the bootstrap analysis of 100 replicates. The ingroup consists of two clades, one comprising of populations of *R. r. sinicus* from northern India, the

Table 3: Genetic distances of mtDNA for taxa of *Rhinolophus rouxii*.

	Taxa	a	b	c	d	e	f	g	h	i
a	*R. ferrumequinum*, U.K.									
b	*R. rouxii* High Wavy Mtns., S. India	0.121								
c	*R. rouxii* Colva, Goa, S. India	0.089	0.121							
d	*R. rouxii* Matale, Sri Lanka	0.107	0.120	0.039						
e	*R. rouxii* Matale, Sri Lanka	0.098	0.117	0.036	0.011					
f	*R. rouxii* Matale, Sri Lanka	0.109	0.124	0.036	0.011	0.009				
g	*R. rouxii* Matale, Sri Lanka	0.100	0.122	0.041	0.020	0.007	0.017			
h	*R. rouxii* Mussoorie, N. India	0.105	0.133	0.086	0.110	0.105	0.112	0.112		
i	*R. rouxii* Mussoorie, N. India	0.103	0.127	0.086	0.104	0.099	0.106	0.106	0.005	
j	*R. rouxii* Mussoorie, N. India	0.103	0.128	0.088	0.106	0.101	0.108	0.108	0.007	0.001

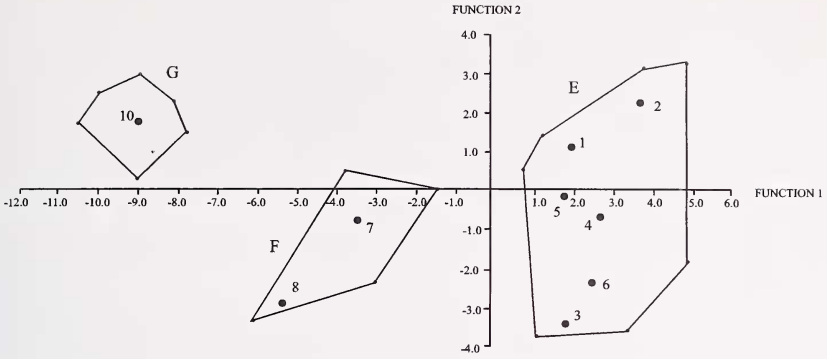

Fig. 3: Ordination of all designated OTU's along the first two discriminant functions from analysis of 57 female specimens. Numbers correspond to OTU means. Lines indicate the extent of scatter of individual specimens. OTU's are listed in Table 1.

other comprising of the remaining taxa. Sequence divergence between the two clades ranges from 8.6–13.3%. The latter clade shows *R. rouxii* from High Wavy Mountains, southern India as sister to a clade comprising *R. rouxii* from Colva, southern India and Matale, Sri Lanka. The individual from Colva is sister to the Sri Lankan clade. Between the Sri Lankan and southern Indian populations, sequence divergence is 3.6–4.1% for the Colva population and 11.7–12.4% for the

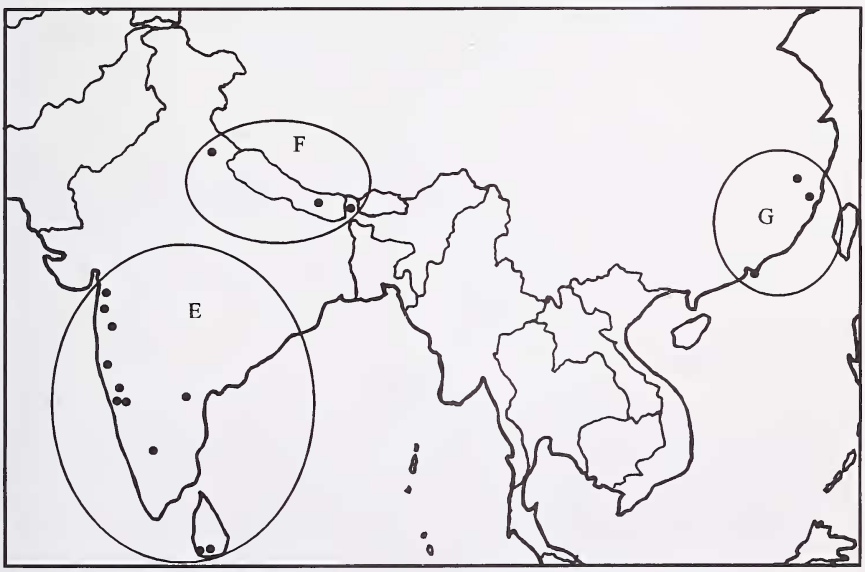

Fig. 4: Geographical representation of clusters E-G from discriminant analysis of female individuals.

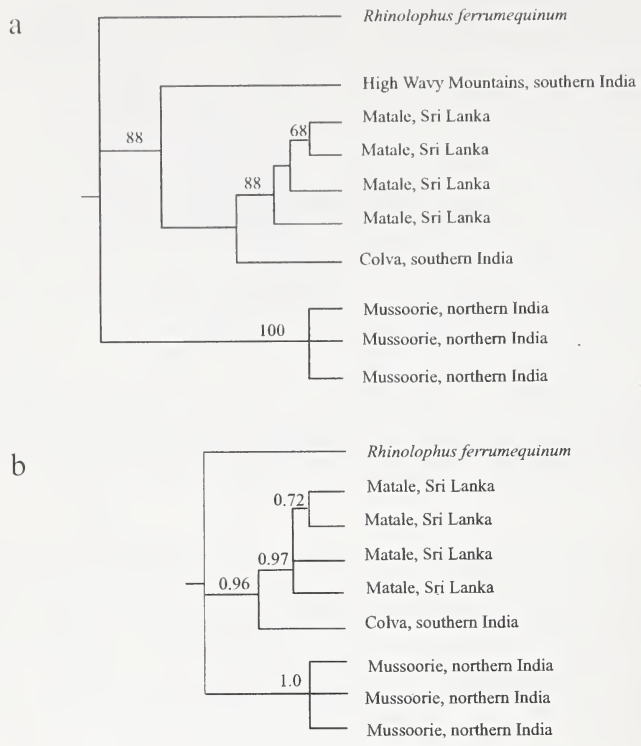

Fig. 5a/b: (a) Strict consensus of 2 most parsimonious trees generated by exact analysis of sequence data. Numbers on branches represent bootstrap node confidence values from 100 replications. (b) Jac support tree. Numbers on branches represent confidence frequencies in nodes as quantified by parsimony jacknifing with Jac (Farris 1995).

High Wavy Mountain population. The values for the Jac (Farris 1996) tree are shown in Fig. 5b. Tree topology supports the results obtained, showing high confidence frequencies for all nodes.

Description of species

Rhinolophus sinicus (Andersen, 1905a)
Chinese horseshoe bat

Rhinolophus rouxii sinicus Andersen 1905a: 98; Chinteh, Anhwei, China.

External characters (measurements included in Table 4): A medium-sized Rhinolophid, with an average forearm length of 47.4 mm (range 45.5–50.0 mm). The ears average 17.8 mm in length (15.8–20.0 mm), being smaller than those of *R. rouxii*. The noseleaf is shorter and narrower than in *R. rouxii*, averaging 12.5 mm in maximum height and 7.7 mm in maximum width. The lancet is broad and short with a well-defined tip (Fig 6a). The base of the sella is broad. In side view, the superior connecting process of the sella is bluntly rounded off, with the base of the sella projecting slightly forwards and downwards. The wing morphology differs significantly to that of *R. rouxii*. The forearms and metacarpals of *R. sinicus* average 4.7% shorter than in *R. rouxii*. However the phalanges, with the exception of the second

Table 4: External, cranial and bacular measurements (mm) of specimens of *R. rouxii* from Sri Lanka, peninsular India and Myanmar and *R. sinicus* from northern India, Nepal and China.

	R. rouxii								*R. sinicus*							
	Sri Lanka				Peninsular India / Myanmar				Himalayan India / Nepal				China / Hong Kong			
	mean	range	s	n	mean	range	s	n	mean	range	s	n	mean	range	s	n
Tail length	26.7	23.0–30.0	1.7	20	26.6	22.0–33.0	2.4	70	25.6	21.5–30.0	2.1	17	26.0	26.0–26.0	–	1
Hind foot length	9.7	9.0–11.0	0.6	22	11.2	7.2–12.8	0.7	74	8.6	7.5–10.0	0.7	19	9.0	9.0– 9.0	–	1
Tibia length	21.1	18.8–23.5	0.9	25	22.6	18.9–26.1	1.2	93	19.5	18.1–21.2	0.8	22	19.3	17.7–20.3	0.7	15
Ear length	20.5	20.0–22.0	0.7	19	19.5	14.5–24.0	1.6	72	17.8	15.8–20.0	1.3	17	19.0	19.0–19.0	–	1
Forearm length	47.3	45.0–50.0	1.4	29	48.9	44.6–52.3	1.5	97	47.5	45.8–50.0	1.2	22	47.3	45.5–49.2	1.2	14
Length 5th metacarpal	37.9	36.0–40.0	0.2	29	38.9	35.9–41.2	1.1	93	38.3	36.9–39.8	0.8	22	37.6	35.8–39.0	0.8	15
Length 4th metacarpal	37.7	34.7–40.6	1.2	29	38.7	35.1–41.2	1.1	93	37.1	35.2–38.9	0.8	22	36.4	34.2–37.9	0.9	15
Length 3rd metacarpal	36.9	34.8–38.9	1.1	29	37.8	34.8–40.1	0.9	93	36.3	34.7–38.0	0.8	22	35.1	34.0–36.4	0.8	15
Length 2nd metacarpal	39.1	37.4–41.4	1.1	29	40.3	36.7–43.0	1.1	92	38.6	36.3–40.6	1.1	22	36.6	34.8–39.4	1.2	15
Length 1st phalanx 5th finger	11.3	10.2–12.4	0.5	29	11.8	10.1–13.2	0.6	92	12.3	11.5–13.2	0.5	22	12.3	11.7–12.8	0.4	15
Length 2nd phalanx 5th finger	12.5	11.2–14.1	0.6	29	13.5	9.0–15.5	0.9	92	12.1	11.0–13.2	0.6	22	11.5	10.3–12.5	0.6	15
Length 1st phalanx of 4th finger	10.3	9.5–11.1	0.4	29	10.8	9.2–12.2	0.6	92	11.4	10.7–12.5	0.4	22	11.5	11.1–12.1	0.3	15
Length 2nd phalanx of 4th finger	12.6	11.2–14.4	0.7	29	13.7	11.6–15.3	0.8	91	13.7	11.9–14.9	0.7	22	13.9	12.8–15.1	0.7	15
Length 1st phalanx 3rd finger	14.4	13.0–15.4	0.5	29	14.9	13.3–16.3	0.6	92	15.4	14.5–16.2	0.4	22	15.1	14.4–15.8	0.4	15
Length 2nd phalanx 3rd finger	20.6	18.1–23.1	1.3	29	22.4	18.2–25.0	1.5	91	22.5	21.0–23.6	0.7	21	23.2	21.5–24.6	1.0	15
Greatest length of skull	21.8	20.6–22.8	0.7	17	22.4	20.9–23.6	0.6	76	20.7	20.0–21.5	0.4	15	20.2	19.9–20.7	0.3	9
Condylocanine length	18.7	17.7–19.7	0.6	21	19.2	17.5–20.9	0.6	88	17.7	17.2–18.4	0.3	16	17.1	16.7–17.7	0.3	12
Length of palate	2.4	2.0– 2.7	0.2	24	2.4	2.0– 3.0	0.2	94	2.1	1.9– 2.4	0.1	20	1.8	1.5– 2.1	0.1	13
Width across upper molars	7.8	7.6– 8.1	0.2	24	8.2	7.6– 8.8	0.2	94	8.0	7.6– 8.6	0.3	21	7.6	7.2– 7.8	0.2	10
Length of upper toothrow	8.5	7.7– 9.0	0.4	26	8.6	8.0– 9.2	0.3	94	7.7	7.4– 8.1	0.2	21	7.3	7.0– 7.7	0.2	15
Width across upper canines	5.5	5.1– 6.0	0.3	24	5.8	5.3– 6.3	0.2	94	5.3	4.9– 5.8	0.2	21	5.0	4.8– 5.2	0.1	13
Interorbital breadth	2.3	2.1– 2.5	0.1	24	2.4	2.0– 2.8	0.2	92	2.5	2.3– 2.7	0.1	19	2.6	2.1– 2.8	0.2	13
Zygomatic breadth	10.7	10.1–11.6	0.3	19	11.2	10.5–11.9	0.3	87	10.4	10.0–10.9	0.3	19	10.2	9.6–10.4	0.2	11
Mastoid breadth	9.9	9.4–10.6	0.3	20	10.3	9.6–10.8	0.2	90	9.5	9.3– 9.7	0.1	18	9.3	9.0– 9.5	0.1	13
Basioccipital width	0.9	0.8– 1.1	0.1	18	1.1	0.7– 1.3	0.1	87	1.1	0.9– 1.3	0.1	18	1.0	0.8– 1.2	0.1	13
Length of mandible	14.9	13.8–15.9	0.5	26	15.3	14.0–16.3	0.5	92	13.8	13.4–14.5	0.3	21	13.2	12.7–13.8	0.3	15
Length of lower toothrow	9.2	8.4– 9.9	0.4	26	9.3	8.5–10.0	0.4	92	8.3	8.0– 8.7	0.2	21	7.8	7.1– 8.2	0.3	15
Greatest height of noseleaf	13.8	13.3–14.2	0.4	7	13.6	11.8–15.5	1.2	16	12.7	10.8–13.6	0.7	12	12.2	11.3–13.5	0.7	9
Greatest width of noseleaf	8.9	8.0– 9.9	0.7	7	8.3	7.1– 9.1	0.5	16	7.9	7.5– 8.5	0.4	12	7.4	6.6– 8.0	0.4	9
Greatest length of baculum	2.6	2.5– 2.7	0.1	2	2.2	1.9– 2.5	0.2	5	2.1	1.9– 2.2	0.1	3	–	–	–	–
Greatest width of baculum	0.8	0.8– 0.8	0.02	2	0.7	0.5– 0.9	0.2	5	0.6	0.4– 0.6	0.1	3	–	–	–	–

phalanx of the fifth finger, average 4.5% longer. This wing structure is similar to that of *R. affinis*, although when these two species are found sympatrically, as at Nala Pani Cave in Mussoorie (30.27'N 78.06'E), *R. affinis* is absolutely larger in all respects. The pelage is soft and silky, and is generally rich russet brown on the back and paler on the belly.

Cranial and dental characters: *R. sinicus* has characteristically small cranial measurements, with condylocanine length ranging from 16.7–18.4 mm (Table 4). The skull is narrow, having an average zygomatic breadth of 10.3 mm, and a mastoid breadth of 9.4 mm. The palate is short, its anterior border lies adjacent to the metacone of the first upper molar m^1; it averages 1.9 mm in length (Fig. 7a). This is in contrast to the longer palate of *R. rouxii* which averages 2.4 mm in length. The dentition is not as robust as *rouxii*, having short upper and lower toothrows, averaging 7.5 mm and 8.1 mm respectively. The upper canine is not in contact with the second upper premolar (pm^4) and the first upper premolar (pm^2) is usually situated in the toothrow. The second lower premolar (pm_2) is often displaced from the toothrow, with the first (pm_2) and third (pm_4) premolars in contact.

Baculer morphology: The shaft of the baculum is long, parallel-sided and generally straight, thickening towards the base. The base is slightly expanded, and the tip is simple and unexpanded (Fig. 8a). The average length is 2.1 mm, and the average width 0.5 mm.

Distribution: *R. sinicus* ranges from southern China, through Nepal and into northern India (Fig. 9). Localities include:

India: Himachal Pradesh: Solan (Das 1986); Uttar Pradesh: Mussoorie (Blandford 1888–91; HZM); Dhakuri (Wroughton 1914); West Bengal: Darjeeling (BMNH); Pashok (Sinha 1973); Sikkim: Tashiding (Bhat 1974).

Nepal: Sipuri (Fry 1925); Thankot; Parchung (BMNH); Godavari; Pulchowki (HZM); Num (FMNH).

China: Sichuan Province: Wanhsien; Fujian Province: Yungan; Nanping; Hebei Province: Ichang; Zheijiang Province; Tunglin; Yunnan: Likiang (Allen 1938).

Hong Kong: Lam Tao Island (BMNH).

Variation: At present, all specimens are referred to the nominate race *R. s. sinicus*. However, those from northern India and Nepal average slightly larger in body and skull size than individuals from China, particularly in condylocanine length, the length of the upper and lower toothrows and the length of the mandible. In addition, the noseleaf in individuals from China is slightly shorter and narrower.

Specimens examined: (S) denotes inclusion in discriminant analysis, (D) denotes inclusion in molecular analysis.

India: Darjeeling, BM.21.1.17.2 (S), BM.79.11.21.57; Mussoorie, HZM.22.28154 (S,D), HZM.23.28155, MM.85 (S), MM86 (S), HZM.21.28153 (S,D), BM.79.11.21.146 (S), BM.79.11.21.149.

Nepal: Godavari, HZM.1.16291 (S), HZM.2.16292, HZM.3.16293 (S), HZM.4.16294 (S); HZM.5.162895 (S), HZM.6.16296 (S), HZM.7.16297 (S), HZM.8.16298, HZM.9.16296; Parchung, BM.21.5.1.3 (S); Thankot, BM.22.5.16.6.

China: Ichang, AM.60217; Nanping, AM.47997, AM.48006, AM.48012 (S), AM.48015 (S), AM.48018 (S), AM.48019, AM.48020 (S), AM.56944, AM.56946; Wanhsien, AM.59607 (S); Yungan, AM.60225 (S), AM.84857 (S), AM.84859 (S).

Hong Kong: Lam Tao Island, BM.66.24 (S).

Habits: In China, *R. sinicus* is common over the southern half of the country. In the south-east it is found at relatively low altitudes of up to 200 metres (656 feet), being recorded from Hebei Province, Fujian Province, Zheijiang Province and Sichuan Province. In the south-west, it is found at higher altitudes of up to 2000 metres (6562 feet), such as a series of specimens collected from southern Yunnan (Allen 1938). In northern India and Nepal, it is restricted to higher elevations. It has been collected at an altitude of 500 metres (1625 feet) in Arunachal Pradesh (Lal 1982); 550 metres (1788 feet) in West Bengal (Bhat, 1974); 862 metres (2800 feet) in Nepal (FMNH) and at 2769 metres (9000 feet) in Uttar Pradesh (Wroughton 1914). In Mussoorie, Uttar Pradesh (1910 metres / 6208 feet), individuals were found by the author roosting in a cave with *R. ferrumequinum* and *R. affinis*. In cold regions, *R. sinicus* hibernates during winter (Blanford 1888–91). It is a largely social species, but segregation of males and females occurs when the females are having their young (Allen 1938).

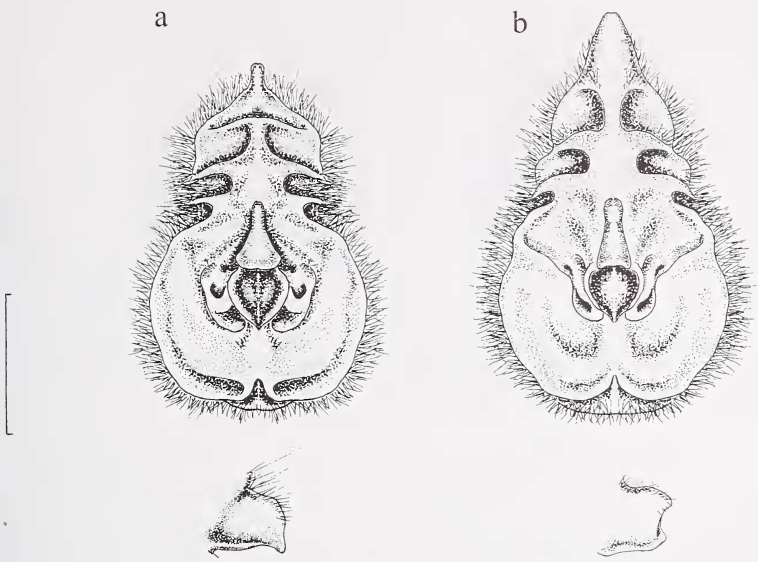

Fig. 6a/b: (a) Noseleaf and sella of *R. sinicus* (HZM.21.28153) from Mussoorie, northern India. Scale = 5 mm. (b) Noseleaf and sella of *R. rouxii* (HZM.11.25681) from Talewadi, southern India. Scale = 5 mm.

Rhinolophus rouxii Temminck, 1835
Rufous horseshoe bat.

Rhinolophus rouxii Temminck, 1835: 30b; Calcutta and Pondicherry, India.
Rhinolophus rubidus Kelaart, 1850: 209; Kaduganava, Sri Lanka.
Rhinolophus fulvidus Blyth, 1851: 182 (error for rubidus Kelaart).
Rhinolophus cinerascens Kelaart, 1852: 13; Fort Frederick, Sri Lanka.
Rhinolophus rammanika Kelaart, 1852: 14; Amanapoora Hill, Kaduganava, Sri Lanka.
Rhinolophus petersii Dobson, 1872: 337; India "precise locality not known".

External characters (measurements included in Table 4): A medium-sized Rhinolophid, with an average forearm length of 48.5 mm (range 44.6–52.3 mm). The ears are larger than in *R. sinicus* averaging 19.7 mm (14.5–24.0 mm). The noseleaf is longer and broader, averaging 13.6 mm in greatest height and 8.5 mm in greatest width. The lancet is tall and narrowly pointed with relatively straight sides (Fig. 6b). The base of the sella is narrow in frontal view. In side view, the superior connecting process of the sella is more rounded than in *R. sinicus*, and the base does not project downwards. In the wing, *R. rouxii* has a longer forearm and longer metacarpals than in *R. sinicus*, however, the phalanges are shorter by an average of 4.5%. The pelage is soft and silky, and ranges from orange to buffy brown. Empirical evidence suggests a seasonal bias in colour with orange and rufous tints predominating from October to April and the paler phases being more common from May to September (Bates & Harrison 1997).

Cranial and dental characters: The skull is more robust than in *R. sinicus*, with condylocanine length averaging 19.1 mm (17.5–20.9 mm) (Table 4). The skull is relatively broad, having a zygomatic breadth averaging 11.1 mm and a mastoid breadth of 10.2 mm. The palate is longer than in *R. sinicus*, its anterior border is at a level of the mesostyle of the first upper molar (m^1); it averages 2.4 mm in length (Fig. 7b). The dentition is relatively robust, having upper and lower toothrows which average 8.6 mm and 9.3 mm respectively. The upper canine is not in contact with the second upper premolar (pm^4) and the first upper premolar

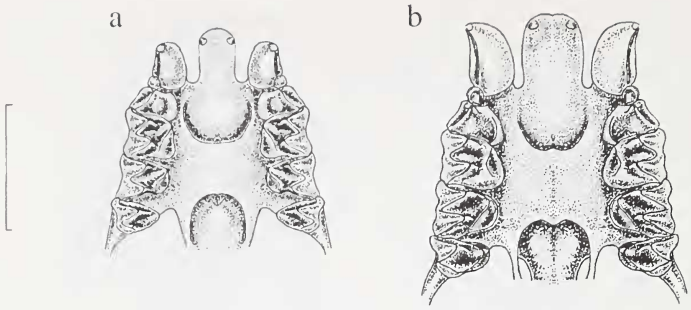

Fig. 7a/b: (a) Right maxillary dentition and palate of *R. sinicus* (HZM.21.28153) from Mussoorie, northern India. (b) Right maxillary dentition and palate of *R. rouxii* (HZM.12.25682) from Talewadi, southern India.

(pm^2) is usually situated in the toothrow. The second lower premolar (pm_3) is usually situated in the toothrow.

Bacular morphology: The shaft of the baculum is long, parallel-sided and generally straight, thickening towards the base, as in *R. sinicus*. The base is slightly expanded, and the tip is simple and unexpanded (Fig. 8b). Bacula examined were found to be slightly longer and broader than in *R. sinicus*, averaging 2.3 mm and 0.7 mm respectively.

Distribution: *R. rouxii* ranges from Sri Lanka, throughout peninsular India to southern Myanmar (Fig. 9). For a full listing of localities see Bates & Harrison (1997 & in press).

Variation (Table 4): Specimens from Sri Lanka are currently referred to *R. rouxii rubidus* (Bates & Harrison 1997). This taxon is smaller in body and skull size than *R. rouxii rouxii* from peninsular India and Myanmar, but not as small as *R. sinicus*. Noseleaf morphology shows Sri Lankan individuals to have, on average, wider noseleaves covering most of the muzzle, whereas in *R. r. rouxii* from peninsular India and Myanmar the noseleaf is relatively narrow. Cranial characters of Sri Lankan individuals also average smaller, particularly condylocanine length, zygomatic and mastoid breadths, width across the canines and mandibular length. The upper and lower toothrows however, are relatively long averaging almost the same in both Indian and Sri Lankan individuals. Although the bacula of Sri Lankan individuals are longer, the morphology is comparable.

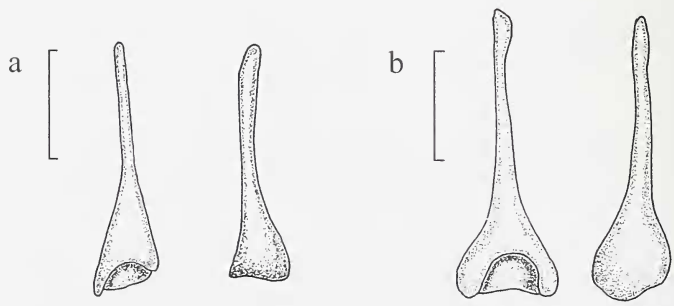

Fig. 8a/b: (a) Baculum (dorsal and right lateral views) of *R. sinicus* (HZM.4.16294) from Godavari, Nepal. Scale = 1 mm. (b) Baculum of *R. rouxii* (IN.62) from Talewadi, India. Scale = 1 mm.

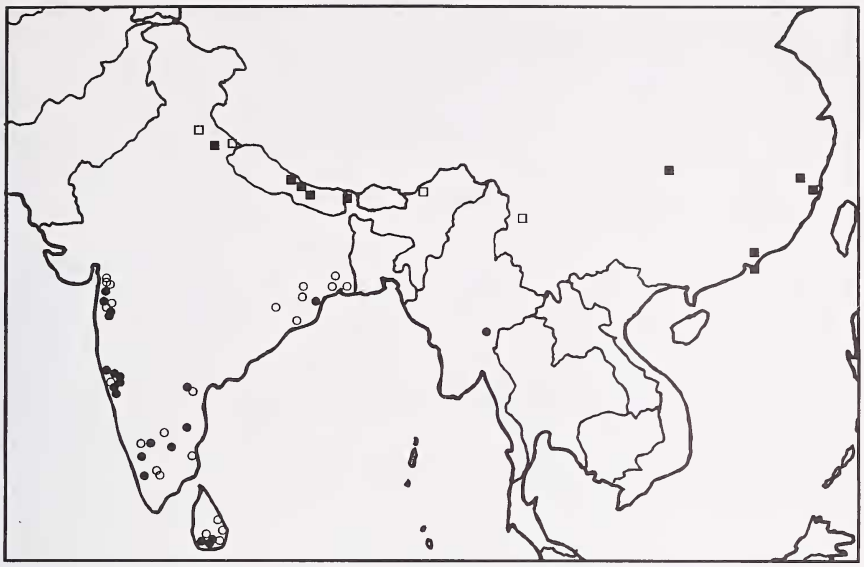

Fig. 9: Distribution map for *R. sinicus* and *R. rouxii* based on specimens examined for this study and additional literature records from Allen (1938) and Bates and Harrison (1997).

Specimens examined: (S) denotes inclusion in discriminant analysis, (D) denotes inclusion in molecular analysis.
Sri Lanka: Ingiriya, HZM.28.28555, HZM.35.28562, HZM.36.28563, HZM.37.28564; Kalutara, BM.20.9.26.2, BM.20.9.26.3 (S), BM.20.9.26.4 (S), BM.20.9.26.5 (S), BM.20.9.26.6, BM.20.9.26.7 (S), BM.20.9.26.8, BM.20.9.26.9 (S), BM.20.9.26.10 (S), BM.20.9.26.11, BM.20.9.26.12 (S), BM.66.5508 (S), BM.66.5509, BM.66.5510, BM.66.5511, BM.66.5512, BM.66.5513, BM.66.5514, BM.6615, BM.66.5516 (S); Matale, HZM.48.29287 (S,D), HZM.54.29330 (S,D), HZM.74 (S,D), HZM.75 (S,D); Monaragala, HZM.29.28556, HZM.32.28559, HZM.33.28560, HZM.40.28567; Pussahena, HZM.16.27473 (S), HZM.17.27474 (S), HZM.18.27475 (S), HZM.19.27476 (S); Ruwanwella, HZM.19.27476 (S), HZM.20.27477 (S); Wellawaya, HZM.38.28565.
India: Asgani, BM.11.7.18.1 (S); Barchi, BM.12.11.28.7 (S); Benhope, BM.25.10.1.2, BM.25.10.1.3; Bombay, CG.1962-345A (S), CG.1962-345B (S), CG.1962-345C (S), CG.1962-345D (S), CG.1962-345E (S), CG.1962-345F (S), CG.1962-345G (S); Coonor, BM.85.3.20.1 (S); Colva, HZM.27.28159 (S, D); Dandeli, BM.12.11.28.6; Devikop, BM.12.6.29.16 (S), BM.12.6.29.17(S), BM.12.6.29.18(S), BM.12.6.29.19 (S); High Wavy Mountains, MM.20 (S), HZM.24.28156 (D), HZM.25.28157, HZM.26.28158 (D), MM.120; Jog Falls, HM.93.18.1 (S), HM.93.18.2 (S), HM.93.18.3 (S), HM.93.18.4 (S), HM.93.18.5 (S), HM.93.18.7 (S), HM.93.18.8 (S), HM.93.18.9 (S); Karnala, CG.1985-1510 (S), CG.1985-1513 (S), CG.1985-1514 (S); Kodura, BM.30.5.24.53 (S), BM.30.5.24.54 (S), BM.30.5.24.55; Mahableshwar, IN.71; Mysore, MM.24 (S), MM.25 (S), MM.30 (S), BM.13.4.11.8, BM.13.4.11.9, BM.13.4.11.10 (S), BM.13.4.11.11, BM.13.4.11.12, BM.13.4.11.13 (S), BM.13.4.11.14, BM.13.4.11.15, BM.13.4.11.16 (S), BM.13.4.11.17, BM.13.4.11.18 (S), BM.13.11.28.7 (S), BM.18.8.3.18, BM.18.8.3.19 (S), BM.18.8.3.20, BM.18.8.3.21 (S),

BM.18.8.3.22; Savantvadi, BM.11.7.18.2 (S), BM.11.7.18.3 (S), BM.11.7.18.4 (S); Shevaroy Hills, BM.30.5.24.44 (S), BM.30.5.24.45 (S), BM.30.5.24.46 (S), BM.30.5.24.47, BM.30.5.24.48 (S), BM.30.5.24.49, BM.30.5.24.50, BM.30.5.24.51 (S); Sirsi, BM.0.4.1.6, BM.0.4.1.7 (S), BM.0.4.1.8 (S), BM.12.11.28.8 (S), BM.12.11.28.9 (S), BM.12.11.28.10, BM.12.11.28.11 (S), BM.12.11.28.12, BM.12.11.28.13 (S); Supa, BM.12.11.28.14 (S), BM.12.11.28.15 (S); Talewadi, HZM.10.25680 (S), HZM.11.25681 (S), HZM.12.25682 (S), IN.61 (S), IN.64 (S), IN.65 (S); Udyagiri, HM.92.86.1 (S), HM.92.86.2 (S), HM.92.86.3 (S), HM.92.86.4 (S), HM.92.86.5 (S), HM.92.86.6 (S), HM.92.86.7 (S), HM.92.86.8 (S), HM.92.86.9 (S), HM.92.86.10 (S).
Myanmar: Toungoo, BM.27.11.18.4 (S).

Habits: *R. rouxii* is a forest species which is restricted to areas with relatively high rainfall. It is common in the Ghats, Kanara and Konkan regions of India (Brosset 1962) and in the lowlands of Sri Lanka (Phillips 1980). *R. rouxii* favours caves and tunnels for diurnal roosting sites, with colony sizes varying from a few individuals to several hundred. *R. rouxii* is often found to roost sympatrically with *Hipposideros speoris* and other species of Hipposiderid bat. Brosset (1962) observed sexual segregation occurring for part of the year, with the males living alone or in small groups and the females gathering in large colonies of several hundred individuals. The diet is probably primarily composed of grasshoppers, moths (Brosset 1962); beetles, termites, mosquitos and other Diptera (Phillips 1980).

Discussion

Analysis of morphometric and DNA sequence data from populations of *R. rouxii* highlights the need for systematic revision within this taxon. In light of the results obtained from morphological and molecular analyses of individuals from Sri Lanka and India, and additional morphological analyses of individuals from Myanmar, Nepal and China, there is sufficient evidence to recognise the Chinese taxon *sinicus* at the specific level. The range of *R. sinicus* is here considered to include the northern India and Nepalese populations on the basis of morpholgical similarities between individuals from these regions and those from China, and the observed sequence divergence between southern Indian and northern Indian populations. At present therefore, populations from China, the Himalayan region of northern India and Nepal are all referred to the nominate form *R. sinicus*. The range of *R. rouxii* is restricted to Sri Lanka, peninsular India and southern Myanmar. This is contrary to the view of Koopman (1993), but follows the taxonomy of Bogdanowicz (1992) who considered *sinicus* to represent a discrete species without comment.

Discriminant analysis suggests that populations from China, Nepal and northern India, and populations from peninsular India, Myanmar and Sri Lanka comprise two well-differentiated taxa. Taxonomic distances also reflect this pattern of variation. Results of the molecular analyses showed mitochondrial DNA sequence of *R. sinicus* from northern India to average 10.9% different to that of *R. rouxii*. Divergence of this magnitude is similar to that found between reproductively and morphologically distinct species of bat, such as within *Pipistrellus* (Barratt et al. 1997) and within the Subfamily Stenoderminae (Van Den Bussche et al. 1993). Populations of *R. sinicus* have an allopatric distribution relative to that of *R. rouxii*. In such cases, when direct proof of reproductive isolation cannot be obtained, it is considered necessary to decide the status of the isolated populations by inference (Mayr & Ashlock 1991). Previous studies examined the taxonomic relationships of a number of species of horseshoe bat, including *R. ferrumequinum* and *R. clivosus*. It was concluded that these taxa represented good species (Thomas 1997). The degree of difference

observed between *sinicus* and *rouxii* in the molecular analyses is comparable to that observed between *R. ferrumequinum* and *R. clivosus*. In the morphometric analyses, *sinicus* and *rouxii* separate more definitively than *ferrumequinum* and *clivosus* (Thomas 1997). These results are comparable with those from other studies, such as Barratt et al. (1997), where species have been designated on the basis of a similar level of sequence divergence.

Within *R. sinicus* there is variation between the Chinese populations and the Indian and Nepalese populations. At present all individuals are referred to the nominate subspecies *R. sinicus sinicus*, however the variation observed suggests that the Indian and Nepalese populations together represent a distinct subspecies, a conclusion supported by examination of morphological characters. However, material from China was not available for use in molecular analyses and as such, it is not considered appropriate to make any formal taxonomic recommendations without examining genetic relationships. Intraspecific variation within *R. sinicus* therefore requires further taxonomic research.

Within *R. rouxii*, variation in morphology was observed between the southern Indian and Sri Lankan individuals which also indicated differentiation at the subspecific level. This variation was not supported by the results of the discriminant analysis, however taxonomic distances clearly separated the Sri Lankan population from those in the High Wavy Mountain region of southern India. The molecular analyses undertaken highlighted substantial diversity within *R. rouxii*. Percentage sequence divergence between the Sri Lankan and Colvan individuals is 3.6–4.1% suggesting a subspecific difference. However, the percentage sequence divergence between the Sri Lankan and High Wavy Mountain populations is 11.7–12.4%, and between the High Wavy Mountain and Colvan popualtions is 12.1%. Due to a lack of material, the variation observed cannot be fully investigated at present, however variation was additionally observed in the discriminant analyses with male individuals from the High Wavy Mountains forming a discrete cluster.

Individuals from Sri Lanka are referred to *R. rouxii rubidus* on the basis of morphological variation, taxonomic distances and the observed sequence divergence between populations in Sri Lanka and mainland India. Until further data are available, individuals from mainland India are referred to the nominate subspecies *R. rouxii rouxii*. This is in agreement with Bates & Harrison (1997).

R. rouxii and *R. sinicus* present an unambiguous case of specific level variation. However, the present study highlights the degree of variation present within species considered by taxonomists to be well defined. Morphometric and molecular analysis of both species has shown there to be a potentially high degree of intraspecific variation present, with possible further divisions at the specific level within *R. rouxii rouxii*. Variation in species with relatively restricted geographical ranges not only has taxonomic implication, but also has implications for conservation. In the "Global Action Plan for Microchiropteran Bats" (Hutson et al. in press), *R. sinicus* and *R. rouxii* are listed as being "lower risk: least concern". At present this is a fair categorisation as both species are relatively widespread throughout their ranges. However, as taxa are split into smaller taxonomic groups, it becomes necessary to establish the ecological requirements of species and subspecies to ensure that they do not become threatened. If for example the population of *R. rouxii* from the High Wavy

Mountains was found to represent a discrete species as suggested by preliminary molecular analyses, its future survival would be dependant on the preservation of a relatively small area of upland forest. However, such potential conservation priorities can only be highlighted if taxonomists are invasive and make full use of modern taxonomic techniques to investigate existing classifications.

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Zusammenfassung

Für eine systematische Revision der Hufeisennase *Rhinolophus rouxii* wurden morphologische Daten externer, cranialer und dentaler Merkmale sowie Sequenzdaten des Cytochrom-b-Gens der mitochondrialen DNA verwendet. Untersucht wurden Individuen der gegenwärtig anerkannten Unterarten *R. rouxii rouxii*, *R. rouxii sinicus* und *R. rouxii rubidus* aus dem gesamten Verbreitungsgebiet. Daten von 22 morphologischen Merkmalen wurden einer multivariaten statistischen Analyse unterzogen. Molekulare Daten wurden mittels Parsimony-Methoden analysiert. Alle Analysen wiesen in hohem Maße darauf hin, daß *R. sinicus* eine eigene Art repräsentiert. Die Population von Sri Lanka wird vorläufig zu *R. rouxii rubidus* gestellt.

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